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Research report

Methotrexate decreases hippocampal cell proliferation and induces memory deficits in rats

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ABSTRACT

Methotrexate (MTX) is a cytostatic agent used in adjuvant chemotherapy for treatment of breast cancer and is associated with cognitive impairment in a subgroup of patients. The aim of this paper is to test whether MTX can rapidly affect various brain structures resulting in decreased hippocampal cell proliferation and white matter damage. We also studied whether cell death occurs in the hippocampus following MTX. All these processes may contribute to the memory deficits reported in patients.

The first study explored the effect of an intravenously injected high-dose MTX (250 mg/kg) on hippocampal cell proliferation, white matter, and cell death. Proliferation was not significantly decreased 1 day after administration of MTX, although a high individual variation was seen. However, 7 days after MTX treatment hippocampal cell proliferation was significantly lower compared to control animals. White matter density was decreased in the lateral corpus callosum of animals treated with MTX, 1 day, 1 week, and 3 weeks after treatment. MTX did not induce hippocampal cell death at any of the time intervals after treatment.

The second study examined the effect of MTX on memory by subjecting animals to a learning task directly followed with MTX treatment. In both learning tasks, memory was impaired in treated animals. In the Morris water maze, animals treated with MTX spent significantly less time in the correct quadrant compared to control animals during the probe trial which was performed 1 week after training and treatment. In contextual fear conditioning, animals treated with MTX showed significantly less freezing behavior compared to control animals, 4 weeks after training and treatment.

These studies suggest that the negative effect of MTX on hippocampal cell proliferation and white matter density may be part of the mechanisms underlying the cognitive impairment observed as side effect after cytotoxic treatment in humans.

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1. Introduction

Adjuvant chemotherapy is frequently applied in the treatment of cancer. The survival rate of patients treated with cytostatics is high; however, the treatment is associated with both short- and long-term side effects. One of the potential long-term effects is cognitive impairment, mostly noticed as a decrease in attention/concentration, speed of information processing and memory [13].

There is an increasing number of human studies exploring the effect of chemotherapy on cognitive impairment [3,13,26,28], while animal studies providing insight in the mechanism behind this phe-

nomenon are scarce. In the few studies performed, methotrexate (MTX) is a frequently used cytostatic. For example, Winocur et al. found impaired learning in BALB/C mice treated with several intraperitoneal injections of MTX and 5-fluorouracil in a Morris water maze, in a non-matching-to-sample test (NMTS), and in a delayed-NMTS of non-spatial memory [38]. Madhyastha et al. found learning impairment in Wistar rats treated with multiple intracerebroventricular MTX injections in a two-compartment conditioned avoidance task (shuttle box test) and altered locomotor and exploratory behavior in a dark-bright arena test [15]. Sieklucka-Dziuba et al. studied the effect of a single intraperitoneal injection of MTX on learning behavior in male and female Albino Swiss mice. Animals treated with MTX showed impaired learning behavior in a passive avoidance task compared to control animals [30].

Our recent study suggests that the effect of MTX on cognition might be mediated by its effects on hippocampal cell proliferation.

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Male Wistar rats were intravenously injected with a high dose of MTX (250 mg/kg) which resulted in a significant decline in performance in a Morris water maze and in a novel object recognition test, 3 and 4 weeks after treatment respectively. We also showed a long-lasting dose-dependent decrease in hippocampal cell proliferation 3 weeks after treatment with MTX [27]. Since hippocampal cell proliferation is thought to play an important role in learning and memory [10,16], the negative long-term effect found in our study may explain the cognitive impairment seen in people treated with adjuvant chemotherapy.

A substantial number of clinical studies observed cognitive impairment several years after completion of chemotherapy treatment [3,13,26,28], but there are also several studies that show the occurrence of cognitive impairment directly after treatment [29,32,34,35] indicating that cytostatics may rapidly affect brain structures involved in cognitive performance. Therefore, in this study, we explored the hypothesis that the inhibitory effect of MTX on hippocampal cell proliferation will show up rapidly after treatment. Since several cytostatics are associated with decreased presence of myelin in white matter and increased cell death [4,7], we also studied the effect of MTX on white matter and cell death. Damage to white matter was explored by measuring the thickness of the corpus callosum, the fiber structure connecting left and right hemispheres. Cell death was studied in the hippocampus using a silver nitrate staining visualizing degenerating neurons [31].

Since damage to the hippocampal area is known to cause retrograde amnesia [17], we expect that the MTX induced lowering of hippocampal cell proliferation on the short-term will affect a previous learned memory. To test this, animals were given two learning tasks (Morris water maze and contextual fear conditioning), after which they were treated with MTX. This study will not only give us insight in the mechanism behind cognitive impairment, it will also give insight in which aspects of learning and memory are affected by MTX.

2. Methods

2.1. General

Adult (3 months of age) male Wistar rats (Harlan, Zeist, the Netherlands, average body weight at the start of the experiment $326 \text{ g} \pm 3.5 \text{ S.E.M.}$) were housed in groups of 3 or 4 animals in clear Plexiglas cages ($58 \text{ cm} \times 38 \text{ cm} \times 20 \text{ cm}$) on a layer of wood shavings with a fixed 12:12 h light:dark cycle (with lights on at 08.00 a.m.) and food and water *ad libitum*. All animals in one cage received the same treatment; meaning that MTX treated animals and control animals were housed in separate groups. Experiments started 2 weeks after arrival of the animals according to the protocol described below. All experiments were approved by the Animal Experimentation Committee of the University of Groningen.

Rats were injected with saline or a high dose of MTX (250 mg/kg, 100 mg/ml solution, Pharmachemie BV, Haarlem, the Netherlands) in the tail vein under a short-lasting (<3 min) mild O_2 –isoflurane anesthesia. After the intravenous MTX injections animals received repeated intraperitoneal injections of the tetrahydrofolate (THFA) calcium leucovorin (10 mg/ml solution, Pharmachemie BV, Haarlem, the Netherlands), which was administered in a protocol similar to the application in patients. Eighteen hours after the injection of MTX, leucovorin was administered in a concentration that was 8% of the injected MTX dose; after 26, 42, and 50 h, the administered concentration was reduced to 4%. Leucovorin is clinically used as a so-called rescue therapy in combination with the cytotoxic agent. Tetrahydrofolate is a co-factor in DNA synthesis; MTX is an inhibitor of the enzyme THFA reductase and depletes the pool of tetrahydrofolates. Leucovorin is a tetrahydrofolate that does not require activation by THFA reductase [5,8]. Pilot studies revealed that leucovorin itself does not have an effect on neurogenesis and that high-dose MTX without leucovorin is lethal, due to severe diarrhea and weight loss.

2.2. Immunohistochemistry

2.2.1. Short-term effect of high-dose MTX on hippocampal cell proliferation

Male Wistar rats were injected with either saline or MTX (250 mg/kg) as previously described. The animals were sacrificed 1 or 7 days after the injection through transcardial perfusion with saline followed by 4% paraformaldehyde. Brains were removed and placed in 30% sucrose solution at 4°C . Microtome sections of the hippocampus ($40 \mu\text{m}$) were stored in 0.01 M PBS including 0.1% azide until immunohistochemical staining.

From the serial sections, every twelfth section from each animal was selected and immunohistochemically stained for Ki-67 using a slightly adapted standard protocol [9]. In brief, free-floating sections were pre-treated with 0.4% H_2O_2 for 30 min, to stop endogenous peroxidase activity. Non-specific binding of immunoreagents was blocked with 3% normal goat serum (Zymed, San Francisco, CA, USA). Subsequently, sections were incubated with mouse-anti-Ki-67 (1:200, MONX10283, Monosan, Uden, the Netherlands) for 48 h at 4°C . After a second blocking step, sections were incubated with a biotinylated secondary antibody (1:400, goat-anti-mouse, Jackson, Wet Grove, PA, USA) for 2 h at room temperature. This was followed by incubation in an avidin biotinylated peroxidase complex (1:400, ABC Elite Kit, Vector Laboratories, Burlingame, CA, USA). Labeled cells were visualized with 0.15 mg/ml diaminobenzidine (DAB) and 0.003% H_2O_2 solution.

After mounting of the sections onto glass slides for microscopic analysis, sections were counterstained with a Mayer-haematoxylin solution for 30 s. Counting of Ki-67 positive cells in both hemispheres of the dentate gyrus was performed under a light microscope with a magnification of $400\times$. Counting was performed in the subgranular layer of the dentate gyrus, the border of the area that was quantified was defined as the subgranular layer having a thickness of two cell diameters. All cells were counted in the subgranular layer of the dentate gyrus from top to bottom of the $40 \mu\text{m}$ thick sections, and counts in both blades were summed. Because every twelfth section of the brain was stained, the number of positive cells was multiplied by 12 for the estimated total amount of Ki-67 positive cells in the hippocampus.

2.2.2. Effects of high-dose MTX on white matter

Brain sections were also analyzed with the Quantimet 550 IW image analysis system (Leica, Cambridge, UK) for changes in white matter. White matter damage was not only studied 1 and 7 days after MTX treatment but also 3 weeks after MTX administration. For this last group sections were used from a previous experiment [27]. From each animal, thickness of the corpus callosum was measured in the middle and 3.5 mm bilaterally of a section at Bregma -3.30 mm .

2.2.3. Effects of high-dose MTX on cell death

Every twelfth section from serial sections of animals sacrificed 1 day, 1 week, or 3 weeks after treatment with saline or MTX was selected and immunohistochemically stained with silver nitrate using a slightly adapted standard protocol [33]. In brief, free-floating slices were rinsed in 4.5% sodium hydroxide/0.6% ammonium nitrate solution. After rinsing, the slices were impregnated for 10 min with a mixture of 7% sodium hydroxide, 8% ammonium nitrate, and 50% silver nitrate. After rinsing the slices in a mixture of 0.5% sodium carbonate and 0.012% ammonium nitrate, development took place with 0.006% ammonium thiosulfate mixed with developer mix, after which the slices were fixed with 37.5% sodium thiosulfate silver fixation solution. The sections were rinsed with distilled water and mounted onto glass slides. Analysis of cell death was performed under a light microscope by scoring the number of cells that had absorbed the silver nitrate, which is considered to be a measure for cell death [31,33].

2.3. Behavioral tests

2.3.1. Morris water maze

The Morris water maze was performed in a circular black pool ($\varnothing 140 \text{ cm}$) with a black platform. The pool was filled with water, with a temperature of $26 \pm 1^\circ\text{C}$, in such a way that the platform was approximately 1 cm below the water surface. The pool was surrounded with external constant cues, and the observer was always positioned at the same location. The task consisted of 4 training days with 4 trials per day, with an intertrial time of 1 h. One trial lasted for 3 min or until the rat found the platform and sat on it for 10 s. If a rat did not find the platform within 3 min it was guided by hand. Animals were divided into two groups based on learning behavior on the last day of the MWM and were injected with either saline or MTX (250 mg/kg) within 1 h after the last trial as previously described.

Body weight of all animals was measured on a daily basis. Body weight gain will be expressed as the percentage of the body weight on the day of the injection. One week after the injection, the animals were placed in the pool without the platform for 1 min (probe trial).

Behavior of the animal was tracked by using Ethovision 3.0 and analyzed for escape latency in the learning phase, being the time from the beginning of the trial until the rat sat on the platform. During the probe trial the time the animal spent in the right quadrant, average swim speed and total distance moved was analyzed.

2.3.2. Contextual fear conditioning

The contextual fear conditioning task was performed with a different group of animals than the Morris water maze. The test was performed in a passive avoidance box composed of black Plexiglas ($42 \text{ cm} \times 42 \text{ cm} \times 42 \text{ cm}$) with a grid floor to establish a dark safe area, with an open top for observation. One of the walls contained a sliding door. On the other side of this door, a small Plexiglas platform ($30 \text{ cm} \times 10 \text{ cm}$) was attached; this platform was lit to form a light, unsafe area. The construction was placed in such a way that the platform was located 70 cm above the floor of the experimental room, while the closed black area was situated on a table. The animals were given 1 habituation session per day for 2 days, to explore the light and dark area freely. Each session lasted 3 min and started on the lit platform. On the third day the door was closed after the animal entered the dark compartment and a foot shock

was given of 0.8 mA for 3 s. After the shock the animals remained in the dark area for 30 s before they were placed back into their home cage. The animals received either saline or MTX (250 mg/kg), as previously described, within 1 h after the foot shock. One month after the foot shock and treatment the animals were placed in the dark compartment for 3 min, with the door closed. The session was recorded and analyzed for immobility behavior.

2.4. Statistics

Body weight was analyzed using repeated measures ANOVA. Light-microscopic counts of Ki-67 positive cells, the density of white matter, and the behavioral tasks were analyzed using one-way ANOVA with treatment as between-subject variable. LSD or Tukey post hoc tests were performed when the ANOVA test was significant. For all statistical tests, a probability value less than 0.05 was considered to be statistically significant.

3. Results

3.1. Immunohistochemistry

3.1.1. Short-term effect of high-dose MTX on hippocampal cell proliferation

The total number of Ki-67 positive cells was 5283.0 ± 293.6 S.E.M. for control animals sacrificed 1 day after injection; 4132.0 ± 1093.8 S.E.M. for animals treated with MTX sacrificed 1 day after injection; 5176.8 ± 713.0 S.E.M. for control animals sacrificed 7 days after injection; 2167.5 ± 520.3 S.E.M. for animals treated with MTX sacrificed 7 days after injection (Fig. 1). The total number of Ki-67 positive cells significantly differed between the groups, $F_{3,22} = 3.952$, $P < 0.05$ for one-way ANOVA. Post hoc test revealed that the total number of Ki-67 positive cells of animals sacrificed 7 days after treatment with MTX was significantly lower than both control groups ($P = 0.01$).

3.1.2. Effect of high-dose MTX on white matter

The density of the lateral corpus callosum was $180.28 \mu\text{m} \pm 6.6$ S.E.M. for control animals; $151.37 \mu\text{m} \pm 6.1$ S.E.M. for animals treated with MTX sacrificed 1 day after injection; $148.77 \mu\text{m} \pm 6.9$ S.E.M. for animals treated with MTX sacrificed 7 days after injection; and $163.53 \mu\text{m} \pm 5.2$ S.E.M. for animals treated with MTX sacrificed 3 weeks after injection. The density of the

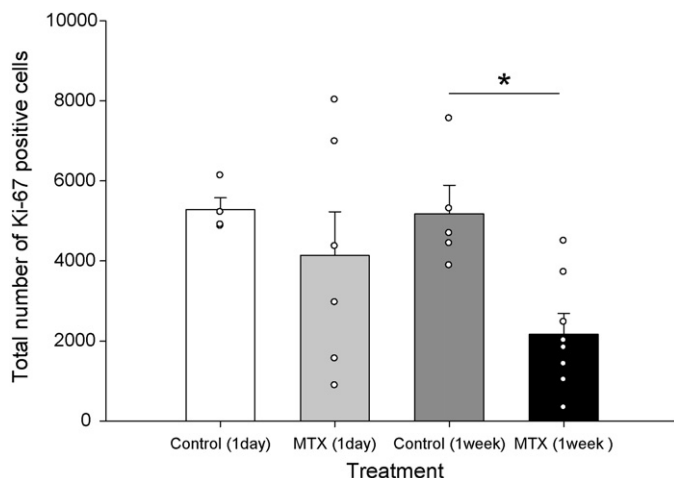


Fig. 1. Total number of Ki-67 positive cells in the hippocampus of control animals, sacrificed 1 day after treatment (open bar, $n = 4$); animals treated with MTX, sacrificed 1 day after treatment (light grey bar, $n = 6$); control animals, sacrificed 1 week after treatment (dark grey bar, $n = 5$); and animals treated with MTX, sacrificed 7 days after treatment (black bar, $n = 8$). Data are represented as mean with standard error of the mean. Scatter plot represents the individual data per group. The total number of Ki-67 positive cells significantly differs between the groups, $F_{3,22} = 3.952$, $P < 0.05$ (one-way ANOVA). The total number of Ki-67 positive cells 7 days after treatment with MTX was significantly lower than both control groups ($P = 0.01$).

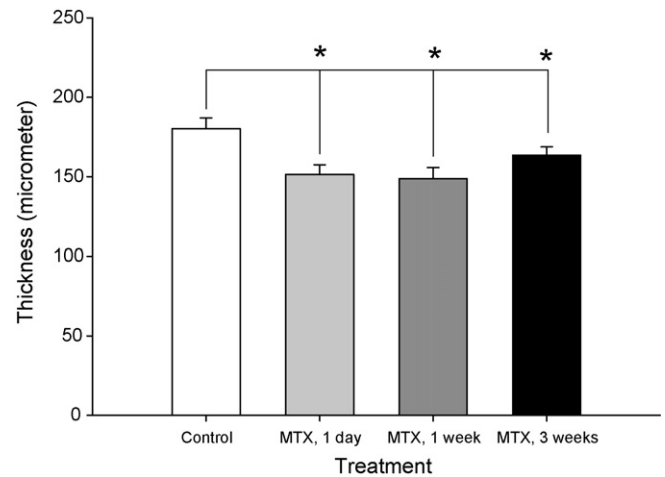


Fig. 2. Density of the lateral corpus callosum, measured above the middle of the left and right hippocampus of control animals (open bar, $n = 12$); animals treated with MTX, sacrificed 1 day after treatment (light grey bar, $n = 7$); 1 week after treatment (dark grey bar, $n = 7$); and 3 weeks after treatment (black bar, $n = 8$). Data are represented as mean with standard error of the mean. The density of the lateral corpus callosum significantly differed between the different groups, $F_{3,31} = 5.547$, $P < 0.005$. Tests for contrasts revealed that the lateral corpus callosum had significantly less density in all MTX treated animals compared to control animals ($P < 0.001$ for all groups).

lateral corpus callosum, was significantly different between the groups, $F_{3,31} = 5.547$, $P < 0.005$ (Fig. 2). Tests for contrasts revealed that the lateral corpus callosum was significantly less dense in all MTX treated animals compared to control animals ($P < 0.001$ for all groups). No effect was seen in the density of the corpus callosum in the center of the brain (data not shown).

3.1.3. Effect of high-dose MTX on cell death

Every twelfth section from animals sacrificed 1 day, 1 week or 3 weeks after treatment with either saline or MTX was selected and stained with silver nitrate to visualize cell death. The number of cells that had absorbed the silver nitrate was very low in all groups and no differences were seen in silver nitrate absorption between the different groups (data not shown).

3.2. Behavioral tests

3.2.1. Morris water maze

The Morris water maze was performed on day –3 to 0, with day 0 being the day of the injection. Body weight gain of the animals was measured on a daily basis (Fig. 3). Body weight gain significantly decreased in rats treated with MTX compared to control animals, when analyzed from day 0 till day 8 ($F_{1,14} = 8.290$, $P < 0.001$). However, at the day of the probe trial (day 7), the decrease in body weight gain was not significant anymore indicating recovery.

Fig. 4A shows the daily average escape latency during the training period of the MWM before treatment. The animals were divided in 2 groups, based on learning behavior on day 4 of the MWM in such a way that there was no difference in learning capacity between the groups to prevent learning bias. Escape latency of both groups significantly improved during the learning phase, with $F_{1,3} = 12.596$, $P < 0.01$ and $F_{1,3} = 15.039$, $P < 0.01$ for control animals and animals treated with MTX respectively. The difference on day 1 did not influence learning behavior of the animals and is caused by random swimming behavior during the first trials.

The animals received a probe trial of 60 s, 1 week after the last trial and treatment. There were no significant differences between the control animals and the animals treated with MTX for average swim speed or total distance traveled (data not shown). However, animals treated with MTX spent significantly less time in the quad-

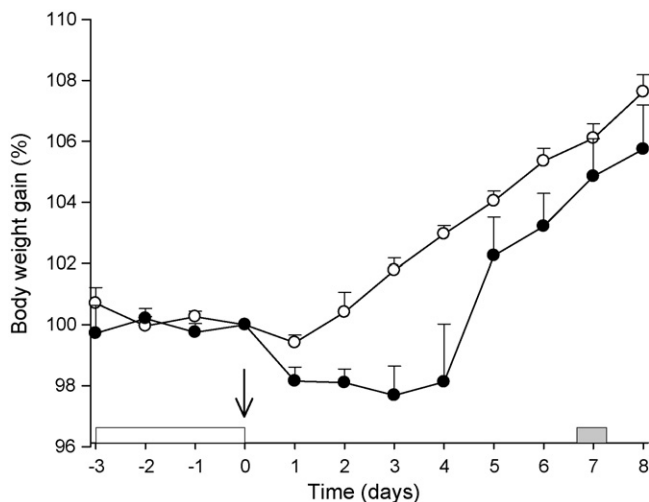


Fig. 3. Body weight gain during and after the Morris water maze for control animals (open circle, $n=8$), and animals treated with MTX (black circle, $n=8$). Body weight on day 0 was set as 100%, bars represent standard error of the mean. The white bar on the x-axis represents the 4 days of the learning phase of the MWM, which lasted from day -3 till 0. The animals were treated with either saline or MTX after the last trial on day 0, which is shown with an arrow. The grey bar represents the probe trial which was given on day 7. Repeated measures ANOVA for day 0 till day 8 revealed a significant difference between the control animals and the animals treated with MTX ($F_{1,13} = 8.290$, $P < 0.001$).

rant where the platform used to be compared to control animals ($33.9\% \pm 2.7$ S.E.M. for control animals and $25.0\% \pm 3.2$ S.E.M. for animals treated with MTX), $F_{1,14} = 5.466$, $P < 0.05$ (Fig. 4B).

3.2.2. Contextual fear conditioning

Contextual fear was tested 1 month after the foot shock and treatment by placing the animals in the dark compartment where they had received the foot shock. Animals treated with MTX showed significantly less freezing behavior compared to control animals ($59.9\% \pm 8.8$ S.E.M. for control animals and $20.4\% \pm 5.7$ S.E.M. for animals treated with MTX), $F_{1,15} = 16.290$, $P = 0.001$ (Fig. 5).

4. Discussion

This study investigated the effects of methotrexate on hippocampal cell proliferation, white matter, and cell death on several time points after MTX administration. Memory performance was tested in 2 different behavioral tasks. The results show that hippocampal cell proliferation is significantly reduced 7 days after treatment with MTX compared to control animals. No significant group effect was seen in animals sacrificed 1 day after treatment. However, the large standard error of the data obtained at 1 day after treatment suggests a large individual differentiation. Indeed, some animals treated with MTX had an equal number of Ki-67 positive cells compared to control animals ($n=2$), whereas others showed a major decrease in the number of Ki-67 positive cells ($n=4$). This finding suggests that there is an individual difference in the speed of response to MTX, resulting in a general decrease in hippocampal cell proliferation 1 week after treatment. Furthermore, treatment with MTX decreased the thickness of the lateral corpus callosum shortly after treatment (1 day) as well as on the long term (3 weeks after treatment). No cell death was noticed after treatment with MTX at any time point studied (1 day, 1 week or 3 weeks after treatment).

A short-term effect of a chemotherapeutic drug on hippocampal cell proliferation was also found in a study of Mignone and Weber in C57BL/6j mice. The animals received three daily injections of either thioTEPA or 5-fluorouracil (5-FU), and the last injection was directly followed by an injection of BrdU. Two hours after the BrdU injection,

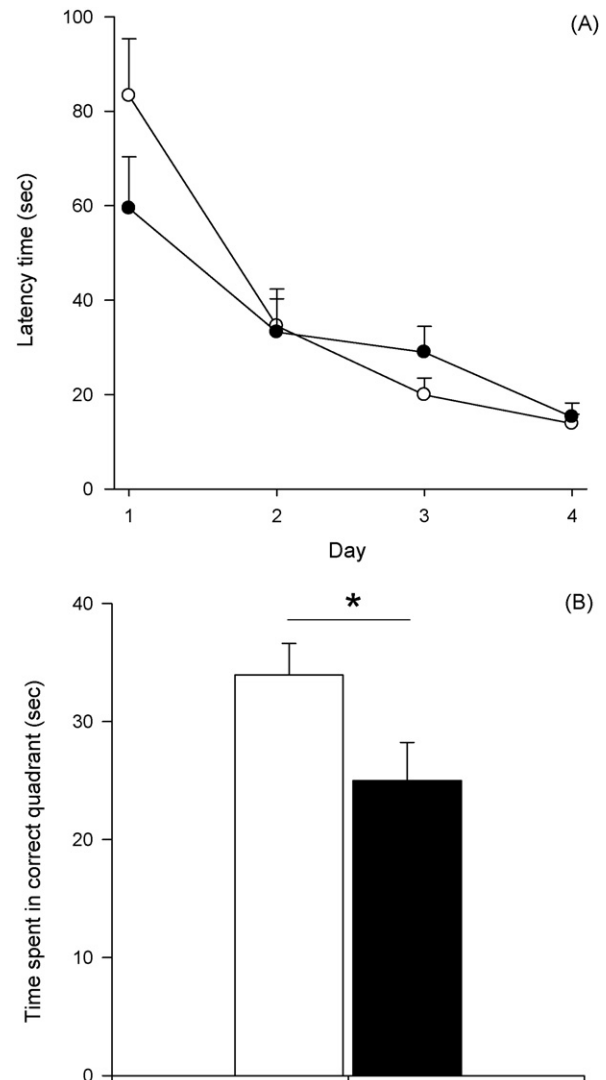


Fig. 4. Results from the Morris water maze for control animals (open bar, $n=8$) and animals treated with MTX (black bar, $n=8$). (A) The mean escape latency and (B) the results from the probe trial. (A) The 4 trials per day are shown as mean escape latency with standard error of the mean. Control animals and animals treated with MTX both significantly improved during the learning phase, with $F_{1,3} = 12.596$, $P < 0.01$ and $F_{1,3} = 15.039$, $P < 0.01$ respectively. There were no significant differences between the groups before treatment. The difference between the control animals and the animals treated with MTX on day 1 is the result of random swimming behavior during the first trials and is not significant. (B) Percentage of time spent in the quadrant where the platform used to be during the probe trial of the Morris water maze, 7 days after the last training day and treatment. Data are represented as mean with the standard error of the mean. Animals treated with MTX spent significantly less time in the quadrant where the platform used to be compared to the control animals treated, with $F_{1,14} = 5.466$, $P < 0.05$.

the mice were sacrificed and the effect of the cytostatic agents on cell proliferation was studied. ThioTEPA significantly decreased hippocampal cell proliferation in a dose-dependent manner, whereas 5-FU did not induce an effect, possibly due to insufficient penetration in the brain [19].

The effect of cytostatic drugs on hippocampal cell proliferation, white matter density, and cell death was also studied by Noble et al. [4,7]. CBA mice received 3 injections for 3 consecutive days of either BCNU or cisplatin. The animals were sacrificed 1 day, 10 days, or 42 days after the last injection. A different group of animals received three injections of 5-FU every other day, and were sacrificed 1, 7, 14, 56 days or 6 months after the last injection. BCNU significantly decreased cell division up to 42 days after treatment.

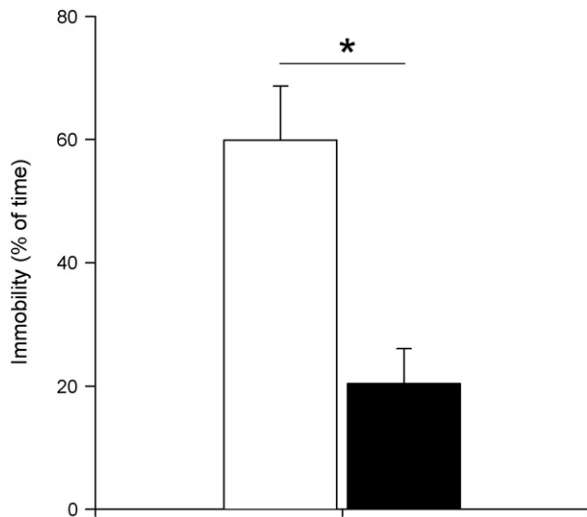


Fig. 5. Percentage of time spent on immobility behavior in the dark compartment of the contextual fear conditioning task 1 month after the foot shock and the treatment. Data are represented as mean with the standard error of the mean. The control animals (open bar, $n=8$) showed significantly more freezing behavior than the animals treated with MTX (black bar, $n=8$), with $F_{1,15} = 16.290$, $P=0.001$.

Cisplatin showed similar effects, but the number of dividing cells returned to normal levels in the subventricular zone and the dentate gyrus 6 weeks after treatment. 5-FU also decreases cell division for up to 6 months in the subventricular zone, for up to 56 days in the corpus callosum, and only on the long term (starting at day 14) in the dentate gyrus. Cell death was measured with a TUNEL staining and all cytostatic drugs induced cell death up to 10 days for BCNU and cisplatin and 14 days for 5-FU. A decrease in myelin sheaths and a deregulation of Olig2 expression, crucial for generating functional oligodendrocytes, was found in the corpus callosum of animals treated with 5-FU which suggest that 5-FU causes damage to white matter [4,7].

Several papers of Palmer et al. also show radiation negatively affects neurogenesis and induces cognitive impairment. Radiation increased the presence of reactive oxygen species (ROS) and induced increased apoptosis in primary neural precursor cells of the rat [14]. Radiation has also been shown to decrease the number of proliferation cells in the hippocampus of C57/BL/J6 mice [20] and to inhibit neural precursor cell proliferation [21]. Furthermore, radiation alters the cell fate profile, resulting in a decrease of neuron production, and an increase in oligodendrocyte production and in proliferation of activated microglia [21].

We also showed in this paper with the results from the behavioral tests that MTX affects memory when given directly after the learning task, shown in the Morris water maze and contextual fear conditioning. The impaired learning caused by MTX could be caused by consolidation deficits or through retrograde amnesia. The memory process consists of four different stages, being encoding, storage, consolidation, and retrieval [1,23,24]. Short-term consolidation can be affected by treating animals directly after training with, for example, convulsive shocks, brain stimulation or drugs. The memory trace is not stored into long-term memory when the consolidation process is disrupted [23]. Since we treated animals with MTX directly after the training period it is likely that we disrupted the consolidation process. This is supported by a number of articles [1,12,23]. Riedel et al. performed a study to see how the consolidation process of a memory trace could be impaired. Male Lister hooded rats were subjected to a Morris water maze and glutamatergic synaptic transmission was blocked at different time points. When neural activity was blocked chronically for 7 days, with the start of the treatment either directly after training or 5 days

later, consolidation of the memory trace was blocked. This suggests that consolidation of a memory trace takes a certain amount of time and that neural activity is necessary for all stages of the memory process [23].

Similar effects were shown in a study by Kinney et al. In this paper, male Sprague–Dawley rats, implanted with a cannula in the left lateral ventricle, were subjected to a Morris water maze with administration of galanin at different time points. Galanin is a neuropeptide that inhibits the release of several neurotransmitters, for example glutamate, norepinephrine, serotonin, and acetylcholine. When the substance was given 30 min or 3 h after the training, it disrupted the consolidation of the memory trace [12].

Retrograde amnesia is the loss of memories acquired before the onset of amnesia and is frequently associated with the consolidation process, since it often affects memories close to the time of the amnesia inducing incident, with sparing of remote memories [18,36]. Winocur examined the role of the hippocampus on retrograde amnesia. Male Long–Evans rats were given food preference training, which was followed immediately, 2, 5 or 10 days later with a lesion in the dorsal hippocampus. The time between the lesion and the food preference training influenced memory. Animals that received a lesion immediately after the test did not show food preference, whereas animals that received the lesion 10 days later showed equal preference as sham operated animals [37].

Anagnostaras et al. explored the effect of electrolytic lesions of the dorsal hippocampus on remote and recent memories. Female Long–Evans rats received two contextual fear conditioning tasks in 2 different contexts, 50 days apart. The animals received a lesion the day after the second contextual fear conditioning task and were retested 10 days after the surgery. Recent, but not remote, memory was impaired in animals with a lesion compared to control animals, meaning that the animals showed no freezing behavior in the recent context and freezing behavior in the remote context [2].

In our opinion, animals treated with MTX in our study suffered from retrograde amnesia due to decreased hippocampal cell proliferation caused by MTX, since hippocampal cell proliferation is thought to be involved in learning and memory [6,10,11]. Evidence for this hypothesis is provided by many papers describing memory impairment after irradiation which strongly inhibits hippocampal cell proliferation [16,22,25].

Combining our data and the literature, we can conclude that disruption of the hippocampal function, either chemically or surgically, leads to retrograde amnesia and impairs the ability to consolidate a memory. Since cognitive impairment is a side-effect in a sub-group of patients after adjuvant chemotherapy [3,13,26,28], we hypothesize that cytostatics, such as MTX, can also disrupt the hippocampal function. Whether this cognitive impairment is solely caused by the negative effect of MTX on hippocampal cell proliferation or if other mechanisms also play a role needs further exploration.

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References

- [1] Akbari E, Naghdi N, Motamedi F. Functional inactivation of orexin 1 receptors in CA1 region impairs acquisition, consolidation and retrieval in Morris water maze task. *Behav Brain Res* 2006;1:47–52.
- [2] Anagnostaras SG, Maren S, Fanselow MS. Temporally graded retrograde amnesia of contextual fear after hippocampal damage in rats: within-subjects examination. *J Neurosci* 1999;3:1106–14.

- [3] Brezden CB, Phillips KA, Abdolell M, Bunston T, Tannock IF. Cognitive function in breast cancer patients receiving adjuvant chemotherapy. *J Clin Oncol* 2000;14:2695–701.
- [4] Dietrich J, Han R, Yang Y, Mayer-Proschel M, Noble M. CNS progenitor cells and oligodendrocytes are targets of chemotherapeutic agents in vitro and in vivo. *J Biol* 2006;7:22.
- [5] Genestier L, Paillot R, Quemeneur L, Izeradjene K, Revillard JP. Mechanisms of action of methotrexate. *Immunopharmacology* 2000;2–3:247–57.
- [6] Gould E, Beylin A, Tanapat P, Reeves A, Shors TJ. Learning enhances adult neurogenesis in the hippocampal formation. *Nat Neurosci* 1999;3:260–5.
- [7] Han R, Yang YM, Dietrich J, Luebke A, Mayer-Proschel M, Noble M. Systemic 5-fluorouracil treatment causes a syndrome of delayed myelin destruction in the central nervous system. *J Biol* 2008;4:12.
- [8] Huennekens FM. The methotrexate story: a paradigm for development of cancer chemotherapeutic agents. *Adv Enzyme Regul* 1994;397–419.
- [9] Kee N, Sivalingam S, Boonstra R, Wojtowicz JM. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. *J Neurosci Methods* 2002;1:97–105.
- [10] Kempermann G. Why new neurons? Possible functions for adult hippocampal neurogenesis. *J Neurosci* 2002;3:635–8.
- [11] Kempermann G, Wiskott L, Gage FH. Functional significance of adult neurogenesis. *Curr Opin Neurobiol* 2004;2:186–91.
- [12] Kinney JW, Starosta G, Crawley JN. Central galanin administration blocks consolidation of spatial learning. *Neurobiol Learn Mem* 2003;1:42–54.
- [13] Kreukels BP, Schagen SB, Ridderinkhof KR, Boogerd W, Hamburger HL, van Dam FS. Electrophysiological correlates of information processing in breast-cancer patients treated with adjuvant chemotherapy. *Breast Cancer Res Treat* 2005;1:53–61.
- [14] Limoli CL, Giedzinski E, Rola R, Otsuka S, Palmer TD, Fike JR. Radiation response of neural precursor cells: linking cellular sensitivity to cell cycle checkpoints, apoptosis and oxidative stress. *Radiat Res* 2004;1:17–27.
- [15] Madhyastha S, Somayaji SN, Rao MS, Nalini K, Bairy KL. Hippocampal brain amines in methotrexate-induced learning and memory deficit. *Can J Physiol Pharmacol* 2002;11:1076–84.
- [16] Madsen TM, Kristjansen PE, Bolwig TG, Wortwein G. Arrested neuronal proliferation and impaired hippocampal function following fractionated brain irradiation in the adult rat. *Neuroscience* 2003;3:635–42.
- [17] Maren S, Aharonov G, Fanselow MS. Neurotoxic lesions of the dorsal hippocampus and Pavlovian fear conditioning in rats. *Behav Brain Res* 1997;2:261–74.
- [18] Meeter M, Murre JM. Consolidation of long-term memory: evidence and alternatives. *Psychol Bull* 2004;6:843–57.
- [19] Mignone RG, Weber ET. Potent inhibition of cell proliferation in the hippocampal dentate gyrus of mice by the chemotherapeutic drug thioTEPA. *Brain Res* 2006;1:26–9.
- [20] Mizumatsu S, Monje ML, Morhardt DR, Rola R, Palmer TD, Fike JR. Extreme sensitivity of adult neurogenesis to low doses of X-irradiation. *Cancer Res* 2003;14:4021–7.
- [21] Monje ML, Mizumatsu S, Fike JR, Palmer TD. Irradiation induces neural precursor-cell dysfunction. *Nat Med* 2002;9:955–62.
- [22] Raber J, Rola R, LeFevour A, Morhardt D, Curley J, Mizumatsu S, et al. Radiation-induced cognitive impairments are associated with changes in indicators of hippocampal neurogenesis. *Radiat Res* 2004;1:39–47.
- [23] Riedel G, Micheau J, Lam AG, Roloff EL, Martin SJ, Bridge H, et al. Reversible neural inactivation reveals hippocampal participation in several memory processes. *Nat Neurosci* 1999;10:898–905.
- [24] Robertson LT. Memory and the brain. *J Dent Educ* 2002;1:30–42.
- [25] Rola R, Raber J, Rizk A, Otsuka S, VandenBerg SR, Morhardt DR, et al. Radiation-induced impairment of hippocampal neurogenesis is associated with cognitive deficits in young mice. *Exp Neurol* 2004;2:316–30.
- [26] Schagen SB, Muller MJ, Boogerd W, Rosenbrand RM, van RD, Rodenhuis S, et al. Late effects of adjuvant chemotherapy on cognitive function: a follow-up study in breast cancer patients. *Ann Oncol* 2002;9:1387–97.
- [27] Seigers R, Schagen SB, Beerling W, Boogerd W, van TO, van Dam FS, et al. Long-lasting suppression of hippocampal cell proliferation and impaired cognitive performance by methotrexate in the rat. *Behav Brain Res* 2008;2:168–75.
- [28] Shilling V, Jenkins V, Morris R, Deutsch G, Bloomfield D. The effects of adjuvant chemotherapy on cognition in women with breast cancer—preliminary results of an observational longitudinal study. *Breast* 2005;2:142–50.
- [29] Shilling V, Jenkins V. Self-reported cognitive problems in women receiving adjuvant therapy for breast cancer. *Eur J Oncol Nurs* 2007;1:6–15.
- [30] Sieklucka-Dziuba M, Saczonek J, Dziuba J, Kleinrok Z. Central action of some cytostatics—methotrexate (MTX) and doxorubicin (DXR). II. The influence on the seizure activity and the learning and memory processes in mice. *Ann Univ Mariae Curie Skłodowska [Med]* 1998:81–8.
- [31] Tashlykov V, Katz Y, Gazit V, Zohar O, Schreiber S, Pick CG. Apoptotic changes in the cortex and hippocampus following minimal brain trauma in mice. *Brain Res* 2007;1:197–205.
- [32] Tchen N, Juffs HG, Downie FP, Yi QL, Hu H, Chemerynsky I, et al. Cognitive function, fatigue, and menopausal symptoms in women receiving adjuvant chemotherapy for breast cancer. *J Clin Oncol* 2003;22:4175–83.
- [33] Ter Horst GJ, Knolles S, Knigge MF, Krugers HJ, Van De Witte SV, Postema F, et al. Silver staining of traumatized neurons: application of a Gallyas procedure in experimental cerebral hypoxia/ischaemia research. *Neurosci Protoc* 1995;02:01–13.
- [34] Wefel JS, Lenzi R, Theriault RL, Davis RN, Meyers CA. The cognitive sequelae of standard-dose adjuvant chemotherapy in women with breast carcinoma: results of a prospective, randomized, longitudinal trial. *Cancer* 2004;11:2292–9.
- [35] Wieneke MH, Dienst ER. Neuropsychological assessment of cognitive functioning following breast cancer. *Psycho-oncology* 1995;1:61–6.
- [36] Wiig KA, Cooper LN, Bear MF. Temporally graded retrograde amnesia following separate and combined lesions of the perirhinal cortex and fornix in the rat. *Learn Mem* 1996;4:313–25.
- [37] Winocur G. Anterograde and retrograde amnesia in rats with dorsal hippocampal or dorsomedial thalamic lesions. *Behav Brain Res* 1990;2:145–54.
- [38] Winocur G, Vardy J, Binns MA, Kerr L, Tannock I. The effects of the anti-cancer drugs, methotrexate and 5-fluorouracil, on cognitive function in mice. *Pharmacol Biochem Behav* 2006;1:66–75.